

## Gene Transfer for the Prevention or Treatment of Type 1 Diabetes

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The endocrine organs are frequently the targets of autoimmunity due to a breakdown in immune tolerance. Rarely, this occurs in the thymus due to mutations in the autoimmune regulator (AIRE) gene and results in the failure to eliminate thymocytes, which react strongly to self-antigens (insulin and many others). These patients develop the autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy syndrome (APECED). However, most autoimmune diseases appear to result from defects in peripheral mechanisms of tolerance. This tolerance is dependent on the expression by T cells of immunoinhibitory molecules (eg, CTLA-4, PD-1) and regulatory (suppressive) cytokines (eg, transforming growth factor beta [TGF- $\beta$ ] and interleukin 10). Regulatory T cells (Treg or Tr) are also critically important. The Foxp3 gene is essential for the generation of Tr cells, and mutations in this gene result in some of the most severe autoimmune disorders, ie, immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX). Our studies demonstrate that gene transfer of immuno-regulatory molecules can prevent type 1 diabetes (T1DM) and other autoimmune diseases. Nonviral gene therapy is performed by injecting plasmid vectors into tissues (usually skeletal muscle) and applying low-intensity electric currents to create pores in cell membranes; this allows DNA to penetrate into cells (*in vivo* electroporation [EP]). A similar technique is applied to perform DNA vaccination against self-molecules (eg, islet cell antigens) and, when this is combined with appropriate immune ligands, results in the generation of Tr cells. DNA vaccination can restore tolerance and protect against some autoimmune endocrinopathies. *In vivo* EP can also be applied to deliver peptide hormones and many other clinically important protein drugs.

The endocrine organs are the most frequent targets of autoimmunity, which results from a breakdown in either central or peripheral immune tolerance. The mechanisms that regulate tolerance are complex and act at the level of both innate and adaptive immunity. In this discussion, we will primarily consider the mechanisms that regulate adaptive immunity in T lymphocytes, eg, T-helper cells (Th1 and Th2), cytotoxic T lymphocytes (CTLs), and regulatory T cells (Treg or Tr).

In the case of T cells, central tolerance is acquired in the thymus, where thymocytes undergo positive and negative selection. When negative selection fails, there is the release of T cells that are autoreactive and highly likely to cause autoimmunity. There are few autoimmune diseases in which failure of central tolerance can be clearly implicated, but this has been noted in patients with mutations of the AIRE gene that encodes a transcription factor. Such mutations cause the APECED, which is inherited in an autosomal-recessive fashion.<sup>1,2</sup> The AIRE gene controls the expression and presentation of many self-antigens (eg, insulin) in thymic medullary epithelial cells and is critically important for the elimination of self-reactive T cells. In APECED, self-reactive T cells are not properly deleted and there is autoimmunity and inflammation affecting several tissues, including the islets of Langerhans (potentially resulting in T1DM) and other endocrine organs.

However, most autoimmune diseases appear to result from defects in peripheral tolerance.<sup>3</sup> Extra-thymic mechanisms of tolerance are important because not all autoreactive T cells are deleted in the thymus. Indeed, some potentially auto-aggressive T cells (perhaps with low-affinity for self-antigens) are normally released from the thymus and must be kept in check by various mechanisms. This includes the expression of immuno-inhibitory



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molecules by T cells such as CTLA-4 and PD-1, and the production of regulatory cytokines such as TGF- $\beta$  and interleukin 10 (IL-10) by either T cells or other cells.<sup>3</sup>

Immunity is further controlled by various types of Tr cells<sup>4-7</sup> that can be broadly divided into 2 subsets:

- natural Tr cells of the CD4+CD25+ phenotype which, in humans, constitute 2%-3% of peripheral CD4+ T cells
- stimulation-induced (or adaptive) Tr cells identified in various models of inflammation, alloreactivity, or autoimmunity.

In contrast to natural Tr cells, induced Tr cells probably differentiate from naïve CD4+CD25-T cells, and act principally by secreting regulatory cytokines, such as TGF- $\beta$ 1 (Th3 cells), or IL-10 and TGF- $\beta$ 1 (Tr1 cells).<sup>8</sup>

Natural CD4+CD25+ Tr cells (released as differentiated Tr cells from the thymus) play an important role in limiting autoimmunity and appear to act by a cytokine-independent, but contact-dependent, mechanism.<sup>4</sup> The gene *Foxp3*, encoding a transcriptional regulator, appears to be necessary for the differentiation of natural Tr and at least some induced Tr cells.<sup>7</sup> A *Foxp3* mutation in scurfy mice results in the absence of these Tr cells, and early death from a multi-organ inflammatory disorder. Mutations in *Foxp3* in humans result in a severe X-linked autoimmune syndrome (termed “IPEX”)<sup>8</sup> (Table 1), usually characterized by enteropathy, polyendocrinopathy (T1DM, thyroid disorders), and eczema. It is apparent early after birth and is one of the most severe autoimmune diseases. These patients develop autoimmune diabetes (T1DM), often within a few days of birth. IPEX is usually fatal in infancy or early childhood and the only effective treatment is bone marrow transplantation, which must be performed at an early age.<sup>8-10</sup>

### CTLA-4 (CD152) and related negative regulatory molecules

The negative regulator CTLA-4 is the best studied and possibly the most potent T-cell inhibitory molecule.<sup>3</sup> It is expressed by T cells after activation and, like the positive co-stimulatory molecule CD28, binds to B7-1 (CD80) and B7-2 (CD86) on the membranes of antigen-presenting cells (APCs). It then downregulates T-cell reactivity by mechanisms that are not fully elucidated. Of note, CTLA-4 delivers a signal early after T-cell activation that primes T cells for responsiveness to TGF- $\beta$  and, which, also appears to be essential for *Foxp3* expression and Tr differentiation.<sup>11,12</sup> Thus, it appears that CTLA-4, TGF- $\beta$ , and *Foxp3* are molecules that work together in the generation of induced Tr cells. Their importance is highlighted by the fact that knockout of any of these three genes in mice results in early death (at 3 or 4 weeks of age) from multi-organ inflammatory disease (Table 1).<sup>3</sup> CTLA-4 is under-expressed in autoimmune diabetes-prone non-obese diabetic (NOD) mice. In humans, some polymorphisms of the gene that alter CTLA-4 levels or function, increase susceptibility to T1DM and other autoimmune diseases.<sup>3</sup>

**Table 1: Multi-organ inflammatory diseases associated with failed tolerance induction**

| Deficient protein (species <sup>a</sup> ) | Disease name | Target tissues of autoimmunity              | Tolerance defect                          |
|---|--------------|---|---|
| AIRE (h, m)                               | APECED       | multi-organ (polyendocrinopathies)          | central, thymus (reduced clonal deletion) |
| TGF- $\beta$ 1 (m)                        | gene KO      | multi-organ (early death)                   | peripheral (Tr and other cells)           |
| CTLA-4 (m)                                | gene KO      | multi-organ (early death)                   | peripheral (T cell hyperactivity)         |
| Foxp3 (h, m)                              | IPEX         | multi-organ (early death) (very early T1DM) | peripheral (lack of Tr cells)             |

m = mouse; h = human

AIRE = autoimmune regulator (transcription factor)

APECED = autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy syndrome;

CTLA-4 = cytotoxic T lymphocyte antigen 4 (also denoted CD152);

IPEX = immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome;

Foxp3 = Forkhead box p3 (transcription factor);

T1DM = type 1 diabetes mellitus.

### Gene therapy for autoimmune diseases

In recent years, there has been intense interest in developing gene therapy approaches for endocrine disease. The success of gene therapy depends on the efficient insertion of genes into the appropriate target cells, without causing cell injury, oncogenic mutation, or inflammation. It should also be possible to re-administer the vector several times, especially in the treatment of chronic diseases. Few vector technologies meet all these requirements. Although the majority of gene therapy studies have been performed with viral vectors, they have serious limitations in terms of immunogenicity and pathogenicity. Nonviral (primarily plasmid-based) gene therapy raises fewer safety concerns and is not hampered by vector immunogenicity, permitting re-administration of the vector. A major limitation of nonviral gene therapy has been low transfection efficiency, but this can be ameliorated sufficiently to rival viral vectors in many applications. One of the most versatile and efficient methods of enhancing gene transfer involves the application of electric field pulses after the injection of nucleic acids (DNA, RNA, and/or oligonucleotides) into tissues.<sup>13</sup> This approach is frequently denoted *in vivo* EP and results in a transient increase in membrane permeability, presumably through the formation of membrane pores, allowing direct entry of macromolecules.

### Nonviral gene therapy vectors

Almost all the nonviral vectors employed thus far are expression plasmids, which have been designed for high expression in striated muscle cells or other cells.<sup>13</sup> The construction of these vectors is quite simple and straightforward. The best plasmids carry a strong promoter (most often the human cytomegalovirus [CMV] immediate-early enhancer promoter [IE-EP]), an intron (such as

CMV intron A), a multiple cloning site for insertion of the gene of interest, and an appropriate transcriptional terminator segment. The CMV IE-EP can be replaced by an endogenous tissue-specific promoter (eg, a muscle-specific promoter).<sup>13</sup> This has the advantage of restricting expression of the vector to a specific tissue. Furthermore, viral promoters are sensitive to cytokines and may be rapidly turned off in the presence of inflammation, while tissue-specific promoters are usually insensitive to inflammation and can be expressed for months or years.

Gene transfer can be accomplished in almost any tissue by EP, but muscle is by far the favourite target.<sup>13-16</sup> Indeed, plasmid delivery into muscle (usually by needle injection), in conjunction with EP, allows the muscle to be used as a bioreactor for the persistent long-term production and secretion of proteins into the blood stream. EP can be applied through electrodes implanted into muscle (or sometimes applied to the skin) at the site of DNA injection. Following entry into the cell, a fraction of the injected plasmid vectors reaches the nucleus, where vector-driven transcription occurs.

Recent review articles address the technical aspects of EP-enhanced nonviral gene therapy.<sup>13-18</sup> After *in vivo* gene electrotransfer into skeletal muscle, the duration of gene expression can range from several weeks to well over a year, depending on the construct and species. More than 300 studies using direct intramuscular (IM) injection of plasmid, followed by EP, provide evidence that adequate levels of secreted (and, in some cases, intracellular) proteins can be achieved using plasmids in a simple, safe, and efficient manner, with significant potential for gene transfer and vaccination for large animals and humans.

### Potential hazards

The safety of DNA administered by injection into muscle has been evaluated in humans and in many animal species.<sup>13</sup> All results indicate that plasmid DNA is generally well-tolerated and that there are no serious adverse effects associated with either plasmid backbones or different active expression cassettes. Some adverse effects, however, include muscle contraction at the time of current application and local pain, which is usually not severe.

One historical concern was that, after injection, the DNA would integrate into the recipient host's chromosomes and lead to mutagenesis and, potentially, insertion carcinogenesis. Animal studies involving plasmid DNA injections have revealed that mutations from a potential integration event would be extremely infrequent, about 3000 times lower than the spontaneous mutation rate for mammalian genomes.<sup>13</sup> However, in the case of DNA vaccination against foreign antigens, it is of some concern that transfected muscle cells may be attacked and injured by the immune system, and this has been reported.<sup>13-15</sup> A related concern is the production of pathogenic anti-DNA antibodies, potentially induced by plasmid DNA and its immuno-stimulatory sequences (ISS), but the risk

appears relatively small. ISS consist of unmethylated CpG-containing bacterial DNA sequences, which bind to the Toll-like receptor 9 (TLR9) of lymphoid cells and activate innate immune responses.<sup>19,20</sup> B cells have mechanisms that prevent autoantibody production in response to CpG stimulation, although this tolerance can be broken. In lupus-prone mice, anti-dsDNA antibody titers are increased by plasmid DNA administration. However, there have been contradictory reports regarding the effects on disease. Some authors have reported that injection of bacterial DNA (carrying CpG-ISS) in lupus-prone mice reduced the severity of disease, while others have reported detrimental effects.<sup>13</sup> Evidently, the effects of CpG motifs on lupus should be analyzed further, and special caution should be exercised in administering CpG-bearing plasmids to patients with autoimmune diseases.

### Therapeutic uses of *in vivo* EP in endocrine diseases

#### *Cytokine inhibitors in autoimmune diabetes*

The transfer of cDNA encoding cytokine inhibitors protects against several autoimmune diseases.<sup>14,15</sup> IL-12 and interferon (IFN)- $\gamma$  are usually detrimental in autoimmune diseases and, consequently, their neutralization is likely to be protective. These 2 cytokines are functionally related, since IL-12 induces IFN- $\gamma$  production by T cells and natural killer (NK) cells, while IFN- $\gamma$  mediates or augments many of the effects initiated by IL-12. To neutralize IFN- $\gamma$ , we constructed a plasmid vector that encodes a soluble IFN- $\gamma$  receptor IgG1-Fc fusion protein (IFN- $\gamma$ R/IgG1-Fc).<sup>21,22</sup> *In vivo*, administration of our IFN- $\gamma$ R/IgG1-Fc vector almost completely blocked IFN- $\gamma$  activity. Moreover, this plasmid was protective in either natural or drug-induced models of autoimmune diabetes,<sup>21,22</sup> which is in agreement with the postulated pathogenic role of IFN- $\gamma$ . In each case, therapy reduced the severity of insulinitis and the frequency of diabetes.

#### *Applications of DNA vaccination*

DNA vaccination has been intensely studied as a means of generating immunity against the antigens of infectious agents or tumours.<sup>23-27</sup> This is due to the simplicity, versatility, and safety of the method. In the vast majority of cases, DNA has been delivered in the form of an expression plasmid, either naked or complexed to other molecules, although other types of vectors can be used. Plasmids can be delivered by IM, intradermal (ID)/epidermal, or subcutaneous (SC) injections, or by oral (eg, with bacterial carrier), pulmonary (aerosols), or other routes (eg, vaginal). Plasmid-encoded antigen is presented by bone marrow-derived APCs, which are most likely dendritic cells (DCs). Compared to other methods, the advantage of DNA vaccination is that delivery of the antigen gene can easily be coupled to the delivery of any of a number of genes that modify the immune response. Moreover, antigen presentation occurs through both the

MHC class I or class II restricted pathways, and all arms of the immune response are activated, ie, Th cells, CTLs and humoral immunity.

DNA vaccination has been effective in rodents, but results have been less impressive in large animals and humans. Consequently, many approaches have been investigated to improve these vaccines,<sup>25</sup> and one of the most effective has been *in vivo* EP. Indeed, the application of EP, regardless of the site of injection, should favour the transfection of a greater variety of cells, including APCs. As an additional mechanism, mild tissue damage as may be induced by EP could provoke an influx of APCs, induce danger signals (eg, inflammatory mediators and chemokines), and enhance the release of antigen from injured cells, thereby increasing antigen presentation.

### *DNA vaccination for T1DM*

Although DNA vaccines are usually immunostimulatory – inducing immunity against foreign or even self-antigens (especially of tumours) – they also protect against either experimental autoimmune encephalomyelitis (EAE), T1DM, or other forms of autoimmunity.<sup>23,24</sup> However, both beneficial and detrimental effects have occurred for reasons that were not elucidated. NOD mice spontaneously develop T1DM, which is clearly a T-cell-dependent autoimmune disease.<sup>28</sup> The autoimmune response is directed against several antigens expressed by pancreatic beta cells; of these, however, only insulin (and its precursor peptides) and glutamic acid decarboxylase 65 (GAD65) are the best studied. We and others have performed studies to determine whether DNA vaccination against these islet antigens could be protective.<sup>13,23,24</sup> We found that DNA vaccination against insulin or GAD65 ameliorated disease, especially if the negative regulatory molecule CTLA-4 was engaged at the time of vaccination. Indeed, this procedure induced protective Tr cells that suppress autoimmunity, at least in part by producing TGF- $\beta$ . Indeed, that cytokine has very broad and powerful immunosuppressive effects.<sup>3</sup>

### *Insulin and other gene therapy*

There has been considerable interest in transplanting genetically engineered cells capable of producing insulin for the treatment of insulin-dependent diabetes or, alternatively, using somatic gene therapy to supply insulin. Unfortunately, it has not been possible to design nonendocrine cells that respond physiologically to glucose. However, continuous low-level (or basal) production of insulin could be beneficial in type 1 or 2 diabetic patients, provided hypoglycemia was not induced. We studied a muscle-based gene therapy approach to achieve this in mice.<sup>29</sup> This required engineering proinsulin for processing by nonendocrine cells. The maturation

process of insulin requires the action of two endopeptidases, proprotein convertase (PC). The PC2 and the PC1 or 3 (PC1/3) are specifically expressed in the beta cells of the islets of Langerhans and some neuroendocrine cells. In nonendocrine cells, similar processing can be accomplished by adding furin cleavage sites, which we added to our construct.

We applied our therapy to streptozotocin (STZ)-induced diabetic mice.<sup>29</sup> This required co-delivery of 2 plasmids, 1 encoding a furin-cleavable insulin, and the other, furin. Insulin was further mutated to increase its activity and *in vivo* EP was used to amplify gene transfer. With this approach, we were able to demonstrate processing of proinsulin to the mature form, and release of sufficient active insulin to prevent hyperglycemia. Our therapy resulted in protection against hyperglycemia and a marked increase in plasma levels of proinsulin, mature insulin, and free C-peptide. Nevertheless, the ultimate goal of regulated insulin production will be very difficult to achieve. An alternative approach for the future, however, might be the application of gene therapy to promote islet-cell proliferation or regeneration, and/or to protect islet cells from injury or apoptosis. Some incretin hormones, such as glucagon-like peptide-1 (GLP-1), are suitable for this purpose, as we have found in our recent studies (M. Kumar et al, manuscript submitted).

### *Leptin gene therapy in models of obesity and diabetes*

There has been considerable interest in developing leptin gene therapy for the control of obesity. This can be done with viral vectors, but it is also possible by transferring the leptin gene in muscle, using EP-enhanced methods.<sup>13</sup> In mice treated by EP, elevated serum leptin concentrations (up to 90 ng/mL) have been recorded (a >200-fold increase over control mice). Indeed, electrogene transfer resulted in hyperleptinemia, decreased food intake, and lower body weight. Furthermore, the production of insulin was lowered in treated mice, but their blood glucose remained normal.

### *Gene therapy to promote wound healing*

A major application of EP-based gene therapy might be in the treatment of cutaneous wounds that occur in many clinical settings and are particularly difficult to treat in diabetic patients. The cost of treating poorly healing foot wounds in the United States has been estimated at over \$1 billion per year. Wound-localized electrogene transfer of DNA, encoding either keratinocyte growth factor or TGF- $\beta$ , has been beneficial in diabetic mice.<sup>30,31</sup> Notably, TGF- $\beta$  and EP appeared to act synergistically to promote healing. Since EP has been applied in patients for other purposes, it could likely be

**Table 2: Applications of electroporation (EP)-enhanced gene therapy**

- Autoimmune diseases (cytokines and cytokine receptors/anticytokines, DNA vaccination)
- Cancer therapy (intra-tumoural or systemic delivery of vectors encoding cytokines, suicide genes, etc.)
- Endocrine therapy (GHRH, leptin, insulin, GLP-1, other.)
- Hematopoietic factors (eg, erythropoietin, GM-CSF, FLT3 ligand)
- Hemophilia therapy (Factor VIII or IX)
- Antibodies
- DNA vaccination against infectious agents or cancer antigens (boosted greatly by EP)

applied to promote wound healing and this will undoubtedly be an area of future clinical investigation.

### **Gene therapy with other hormone-encoding plasmids**

Some authors have tested a growth hormone-releasing hormone (GHRH)-expressing plasmid in skeletal muscle following intramuscular injection enhanced by EP.<sup>13,16,32,33</sup> GHRH is released into the systemic circulation and ectopically stimulates the animal's pituitary to produce and release growth hormone (GH). Young pigs, directly injected with as little as 0.1 mg of a GHRH-expressing plasmid, had significantly greater weight gain than controls, significant increases in lean body mass, and decreases in fat mass. The offspring of gilts (250-400 kg) injected IM and electroporated at day 85 of gestation with 1-5 mg of a GHRH-expressing plasmid have optimized growth characteristics because of improved intra-uterine weight gain and enhanced maternal lactation performance.<sup>13,32,33</sup> Thus, the piglets from treated gilts were larger at birth and weaning compared to controls and exhibited a significantly reduced morbidity and mortality. Analysis of >300 treated animals revealed that expression was maintained for at least 1 year and the beneficial effects on offspring occurred for 3 consecutive pregnancies in the treated animals after a single plasmid administration.

The positive results obtained with plasmid-based GHRH in farm animals prove that, by combining adequate plasmid design with the EP method, physiologic levels of a transgene product can be obtained, even in a 500 kg animal, giving hope that this and other applications may translate into a number of human applications.

### **Nonviral gene transfer in humans**

EP has been applied successfully in humans for the intratumoural delivery of some chemotherapeutic agents, such as bleomycin.<sup>13,17</sup> Clinical trials of

EP-enhanced gene therapy of tumours are ongoing, but few results have been published. Most of the human studies of nonviral gene transfer have been in the area of DNA vaccination, although EP was not applied. Notably, immune responses can be generated against malaria antigens using IM injection. DNA vaccination and recent studies point to sequential plasmid/virus genetic immunization strategies (prime-boost) as an effective method of generating immunity.<sup>25</sup> Nonviral DNA transfer into humans has a remarkable safety profile and is, therefore, attracting more attention.

### **Future prospects**

Gene transfer using EP can be effectively applied in both small and large animals and has many potential applications (Table 2). This approach has been successfully employed in preclinical autoimmune and/or inflammatory diseases to deliver either cytokines, anti-inflammatory agents, or mutated co-stimulatory molecules. Numerous studies have demonstrated the effectiveness of intratumoural delivery of therapeutic vectors. Importantly, it has been found to be highly effective in boosting DNA vaccination against a wide variety of antigens, which is of relevance to infection, cancer, or autoimmunity. One of the most promising applications, however, is in the systemic delivery of protein drugs (eg, endocrine hormones, hematopoietic factors, antibodies, enzymes, and others.)

The use of nonviral nucleic acids in experimental therapy is constantly expanding. The most remarkable new development, however, is the introduction of small inhibitory RNA (siRNA)-based therapeutic agents. Indeed, synthetic or vector-delivered siRNAs are powerful new tools for gene silencing and their potential therapeutic applications are numerous. However, targeting the *in vivo* delivery of these molecules to a specific tissue is difficult and EP-enhanced nonviral methods of nucleic acid transfer have advantages in terms of simplicity, effectiveness, and safety.

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